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In vitro antifungal effects of some chemotherapeutic agents against fungi commonly isolated from repeat breeder animals

A. A. Farrag [1], M. A. Ismail [2,*], K. A. Abdel-Razek[1] and A. A. Ali [1]

[1]**Theriogenology Department, Faculty of Vetrinary Medicine, Assiut University**
[2]**Department of Botany, Faculty of Science, Assiut University**

[*]Corresponding author: e-mail: madyismail@yahoo.com
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Abstract: The sensitivity of fungi isolated from the female genital organs with or without clinical signs of farm animals that failed to conceive after being bred with fertile males more than two times, to some antifungal agents was determined. Seven antifungal therapeutic agents were tested against seventeen fungal isolates from repeat breeder cows (6 isolates), buffaloes (6) and mares (5) using the disc diffusion method. The most effective antimycotic agents were nystatin followed by terbinafine, ketoconazole, miconazole, fluconazole and povidine iodine. Griseofulvin, on the other hand, was not effective against any of the fungi tested. The minimum inhibitory concentration of nystatin ranged from 156.25 to 1250 IU/ml (31.0 - 250 µg/ml), terbinafine from 78.125 µg/ml to 5 mg/ml, ketoconazole from 156.25 µg/ml to 5 mg/ml, miconazole from 1.25 to 10 mg/ml and fluconazole from 2.5 to 10 mg/ml. The MICs of povidine iodine (betadine antiseptic) ranged from 50 to 100 mg/ml. Fungal isolates belonging to *Acremonium strictum*, *Aspergillus flavus*, *Emericella nidulans* and *Penicillium chrysogenum* showed high sensitivity to terbinafine at concentration ranging from 78.125 to 156.25 µg/ml. *Candida albicans* was more sensitive to nystatin and ketoconazole than other antifungal agents.

Key words: antifungal therapeutic agents, fungi, cows, buffaloes, mares, repeat breeders.

Introduction

The most prevalently utilized antifungal agents include the polyenes and the azoles. The polyenes are effective by binding to ergosterol, the fungal membrane sterol, resulting in an increased permeability to the cell wall and eventual cell death (Horowitz *et al*. 1987). The polyenes include amphotericin B, nystatin and natamycin (Carter and Chengappa 1995). The azoles function by inhibition of the cytochrome P-450-mediated removal of the C-14 methyl group from the ergosterol precursor, lanosterol (Vanden Bossche *et al*. 1987). The azole derivatives include clotrimazole, econazole, ketoconazole, fluconazole and itraconazole of which fluconazole and itraconazole are members of the triazoles with 3 nitrogen molecules, whereas clotrimazole, miconazole and ketoconazole are imidazoles with 2 nitrogen molecules (Carter and Chengappa 1995).

Griseofulvin was found earlier to have no inhibitory action on yeasts (Aller- Gancedo 1978) or species of *Candida* (Jand *et al*. 1978), however, it was reported to have effect only on dermatophytes (Sandhu and Randhawa 1964, Karaca and Koc 2004). On the other hand, intrauterine infusions or irrigation of animal endometritis and/or cervicitis with povidine-iodine solution showed good results for the recovery and gave negative isolations (Osman and Abou-Gabal 1975, Koujan *et al*. 1996). Betadine solution was reported to have antimycotic activity against *Candida albicans*, *Proactinomyces ligniteresi*, *Aspergillus fumigatus*, *Absidia corymbifera* and *Mucor pusillus* (Kremlev and Banakova 1979) and *Candida albicans*, *Cryptococcus neoformans*, *Cryptococcus uniguttulatus* and *Rhodotorula rubra* (Theraud *et al*. 2004).

Clinical isolates of *Scopulariopsis* spp. (Aguilar *et al*. 1999, Carrillo-Munoz *et al*. 2005), *C. albicans* and *R. rubra* (Barchiesi *et al*. 2000, Carrillo-Munoz *et al*. 2002, Pfaller *et al*. 2004, Mallie *et al*. 2005, Garg *et al*. 2006) showed different responses towards the antifungal agent fluconazole (fungican). The imidazole derivatives (including miconazole and ketoconazole) showed different antimycotic activities against *C. albicans*, *A. flavus*, *A. niger*, *A. fumigatus*, *Emericella nidulans*, *Penicillium* spp., Mucoraceous species (Aller-Gancedo 1978, Odds 1980, Aguilar *et al*. 1999, Barchiesi *et al*. 2000, Sirohi and Khar 2000, Carrillo-Munoz *et al*. 2002, Garg *et al*. 2006, Shams-Ghahfarokhi *et al*. 2006) and *Scopulariopsis brevicaulis* (Aguilar *et al*. 1999).

Nystatin was found to be active against species of *Candida* and *Rhodotorula* (Kucharski and Rozewicka 1974, Saxena and Ishaque 1977, Jand *et al*. 1978, Carrillo-Munoz *et al*. 2002, Pal 2002) and on *Penicillium* sp., *A. flavus*, *A. niger*, *A. fumigatus*, *Aureobasidium* sp., *Rhizomucor pusillus*, *Cladosporium cladosporioides*, *C. albicans*, *C. tropicalis*, *Cryptococcus laurentii*, and *R. rubra* (Sirohi and Khar 2000). Terbinafine (Lamisil) showed also a potent

activity *in vitro* against *A. flavus* and *A. niger*, *Penicillium* spp., *Acremonium* spp., *Cladosporium* spp. and *Alternaria alternata* (Moore *et al*. 2001, Garcia-Effron *et al*. 2004) and was active against fungi from clinical specimens e.g. *Rhizopus microsporus, R. oryzae, A. corymbifera, Cunninghamella bertholletiae, R. pusillus, Mucor ramesissimus* and *M. circinelloides* (Gomez-Lopez *et al*. 2003), *Trichophyton rubrum, T. mentagrophytes, T. tonsurans* and *T. verrucosum* (Karaca and Koc 2004) and *C. albicans* (Garg *et al*. 2006). However, *Scopulariopsis brevicaulis* showed resistance *in vitro* to terbinafine (Garcia-Effron *et al*. 2004).

The present work was designed to study the sensitivity of fungal species isolated from the genital organs of farm animals that failed to conceive after being bred with fertile males more than twice to some antifungal agents. Also, the minimum inhibitory concentration for each antifungal agent was determined.

Materials and Methods

Antimycotic agents used

The trade names, active ingredients, formulations, producing companies and concentrations of the seven antimycotic agents used are shown in Table (1). Each antimycotic agent was dissolved in its suitable solvent according to USPDI (1999). Nystatin, griseofulvin and micronazole were dissolved in dimethyl formamide (DMF) whereas terbinafine, ketoconazole and fluconazole were dissolved in methanol, and povidine iodine solution was diluted in distilled water.

Fungal strains tested

Seventeen fungal strains isolated from the female genital organs with or without clinical signs of farm animals that failed to conceive after being bred with fertile males more than twice were used in this study. Sources of these strains were as follows: 6 from cows, 6 from buffalos and 5 from mares (Table 2), which were isolated in a previous work and deposited in the culture collection of the Assiut University Mycological Centre (AUMC).

Table 1: Antifungal agents used in the sensitivity test

No	Trade name	Scientific name (active ingredient)	Formulation	Concentration	Producing company
1	Betadine	Povidine iodine	Antiseptic solution	10%	Nile Company, Egypt
2	Fungican	Fluconazole	Capsules	150 mg/cap	Amoun Pharmaceutical Company, Egypt
3	Lamisil	Terbinafine	Tablets	125 mg/tab	Novartis Pharma S.A.E.
4	Miconaz	Miconazole nitrate	Powder	2 g/100 g powder	Medical Union Pharmaceutical Company
5	Nizoral	Ketoconazole	Tablets	200 mg/tab	Janssen Cilag
6	Nystatin	Nystatin	Oral drops	100.000 I.U/ml	EIPICO (Egyptian International Pharmaceutical Industries Co.)
7	Ultragriseofulvin	Griseofulvin	Tablets	125 mg/tab	Kahira Pharmaceutical & Chemical Industries Company, Egypt

The sensitivity test

The method described by Cruickshank (1965) was carried out to assess the activity and the minimal inhibitory concentrations of the antifungal agents used. Double-fold serial dilution from each antifungal agent was prepared in its suitable solvents. The filter paper discs were immersed in each concentration of the antifungal solution and left until full saturation. Discs were placed on the surface of Sabouraud dextrose agar (Moss & McQuown, 1969) seeded with the test isolate (5 µl of the fungal suspension prepared by adding 3 ml of sterile distilled water to slant culture). Discs saturated with solvents served as controls. Cultures were incubated at 25°C for 48 hours after which the zones of inhibition of fungal growth around discs were measured in mm (Venugopal and Venugopal 1994). For each fungal isolate the minimum inhibitory concentration (MIC) was determined as the lowest concentration of the antifungal agent that prevents visible fungal growth after its optimal incubation period.

Results and Discussion

Sensitivity of fungal isolates to antifungal agents

Results in Table (2) reveal that nystatin followed by terbinafine and ketoconazole were highly effective on the tested fungal isolates. Nystatin was active against all fungi tested at 1250 I.U/ml (250 µg/ml) concentration. The MIC of nystatin on *Alternaria alternata* (isolated from cow) was 156.25 IU/ml (=31.25 µg/ml) but, on *A. flavus, A. niger, P. chrysogenum* and *Rhodotorula mucilaginosa* (from cows), *Aphanoascus fulvescenes, A. flavus, A. niger, Chrysosporium tropicum* (from buffaloes) and *C. albicans, C. cladosporioides* and *Rhodotorula mucilaginosa* (from mares) was 625 IU/ml (=125 µg/ml). However, the MIC of nystatin was much higher (1250 IU/ml) (=250 µg/ml) on *A. strictum* (from cow), *C. albicans* and *E. nidulans* (from buffaloes) and *P. chrysogenum* and *Scopulariopsis brevicaulis* (from mares). In this respect, Sirohi and Khar (2000) found the MIC of mycostatin on *Penicillium* sp. and *Cryptococcus laurentii* was 25 IU/ml, *Aspergillus niger, A. fumigatus, Aureobasidium* sp., *C. albicans* and *C. tropicalis* was 50 IU/ml and *A. flavus, Rhizomucor pusillus, C. cladosporioides* and *R. rubra* was 100 IU/ml. Besbes *et al.* (2002) found also that nystatin was effective against *S. brevicaulis* from otomycosis. In vitro studies of Kucharski and Rozewicka (1974), Jand *et al*. (1978), Carrillo-Munoz *et al*. (2002), Pal (2002) and Munguia and Daniel (2008) revealed also the sensitivity of *C. albicans* to mycostatin. Also, nystatin at 1.42 µg/ml was active on all strains of *Candida* tested by Aller Gancedo (1978). However, it was slightly inhibitory to *C. albicans* from repeat breeding bovines (Saxena and Ishaque 1977).

Terbinafine (lamisil) at 5 mg/ml was active against all fungi tested in the present study. The MIC of terbinafine on *A. strictum* and *A. flavus* (from cows), *A. flavus* and *E. nidulans* (from buffaloes) and *P. chrysogenum* (from mare) was 78.125 µg/ml, but on *P. chrysogenum* (from cow), *A. fulvescenes, A. niger* and *C. tropicum* (from buffaloes) and *C. cladosporioides* (from mare) was 156.25 µg/ml. However, much higher concentrations of terbinafine were needed to inhibit *A. niger* from cow (312.5 µg/ml), *C. albicans* from buffalo and mare (1.25 µg/ml) and on *A. alternata, R. mucilaginosa* from cows, *R. mucilaginosa* and *Scopulariopsis brevicaulis* from mares (5 mg/ml). In this respect, Garg *et al*. (2006) found terbinafine to be active against *C. albicans* with MIC determined by a macrodilution method ranging from 2-4 µg/ml. On the other hand, Garcia-Effron *et al*. (2004) stated that terbinafine showed a potent activity *in vitro* against *A. flavus* and *A. niger* with MIC ranging from 0.03-4 and 0.06-2 mg/l respectively using broth microdiluion method with modification. Also, the in vitro activity of terbinafine was superior against *A. flavus* and *A. niger* with MICs being determined using a microtiter method on two different media ranging from $\leq$0.03->16, $\leq$0.03->16 and 0.125-1, 0.06-1 µg/l respectively (Moore *et al*. 2001). Terbinafine showed also strong activity *in vitro* against species of *Penicillium, Acremonium, Cladosporium, Scopulariopsis* and *Alternaria* with MIC much lower in all cases than that reported in the current study, where their MICs, respectively, were 0.03-8, 0.06-8, 2, 1-16, and 0.25 mg/L (Garcia-Effron *et al*. 2004). Terbinafine was also found active against other fungi from clinical specimens e.g. *Rhizopus microsporus, R. oryzae, Absidia corymbifera, Cunninghamella bertholletiae, Rhizomucor pusillus, Mucor ramoissimus* and *M. circinelloides* (Gomez-Lopez *et al*. 2003), *Trichophyton rubrum, T. mentagrophytes, T. tonsurans* and *T. verrrucosum* (Karaca and Koc 2004).

Table 2: Minimum inhibitory concentrations (MICs) of antifungal agents tested on fungi commonly isolated from cows, buffalo-cows and mares that failed to conceive.

Fungus	Source	AUMC no.	PI mg/ml	F mg/ml	M mg/ml	K µg/ml	T µg/ml	N IU/ml
Acremonium strictum W. Gams	Cow	3367	100	2.5	5	625	78.125	1250
Alternaria alternate (Fries) Keissler	Cow	3370	100	5	2.5	312.5	5 mg	156.25
Aphanoascus fulvescens (Cooke) Apinis	Buffalo	3376	100	2.5	5	312.5	156.25	625
Aspergillus flavus Link	Buffalo	3375	50	2.5	5	156.25	78.125	625
Aspergillus flavus Link	Cow	3365	100	5	2.5	156.25	78.125	625
Aspergillus niger van Tieghem	Cow	3364	100	5	10	5 mg	312.5	625
Aspergillus niger van Tieghem	Buffalo	3371	100	5	5	1.25 mg	156.25	625
Candida albicans (Robin) Berkhout	Buffalo	3374	50	2.5	5	156.25	1.25 mg	1250
Candida albicans (Robin) Berkhout	Mare	3379	100	2.5	1.25	625	1.25 mg	625
Chrysosporium tropicum Carmichael	Buffalo	3377	100	2.5	1.25	312.5	156.25	625
Cladosporium cladosporoides (Fresenius) de Vries	Mare	3381	50	2.5	2.5	5 mg	156.25	625
Emericella nidulans (Eidam) Vuillemin	Buffalo	3378	50	2.5	1.25	625	78.125	1250
Penicillium chrysogenum Thom	Cow	3368	100	10	2.5	156.25	156.25	625
Penicillium chrysogenum Thom	Mare	3382	50	5	1.25	156.25	78.125	1250
Rhodotorula mucilaginosa (Jorgensen) F.C. Harrison	Cow	3369	100	10	10	1.25 mg	5 mg	625
Rhodotorula mucilaginosa (Jorgensen) F.C. Harrison	Mare	3380	100	10	10	1.25 mg	5 mg	625
Scopulariopsis brevicaulis (Saccardo) Bainier	Mare	3383	100	10	2.5	1.25 mg	5 mg	1250

• AUMC: Assiut University Mycological Centre culture collection, PI: Povidine iodine (mg/ml); F: Fluconazole (mg/ml); M: Miconazole (mg/ml); K: Ketokonazole (µg or mg/ml); T: Terbinafine (Lamisil) (µg or mg/ml); N: Nystatin (IU/ml).

Ketoconazole (Nizoral) was active against all fungi tested at 5 mg/ml concentration (Table 2). The MIC of ketoconazole on *A. flavus* and *P. chrysogenum* (from cows), *A. flavus* and *C. albicans* (from buffaloes) and *P. chrysogenum* (from mare) was 156.25 µg/ml, while on *A. alternata* (from cow) and *A. fulvescenes* and *C. tropicum* (from buffaloes) was 312.5 µg/ml, however, on *A. strictum* (from cow), *E. nidulans* (from buffalo) and *C. albicans* (from mare) was 625 µg/ml. It has been reported that the MIC of ketoconazole on isolates of *C. albicans* ranged between 0.125-64 µg/ml using an agar dilution assay (Shams-Ghahfaroki *et al*. 2006), between 0.031125-16 µg/ml using a macrodilution method (Garg *et al*. 2006) and between ≤0.03-16 µg/ml using a broth microdilution method (Barchiesi *et al*. 2000). In the study of Carrillo-Munoz *et al*. (2002) on 75 clinical isolates of *C. albicans*, 8 strains were resistant, 19 of intermediate susceptibility and 48 susceptible to ketoconazole (using 15µg/ml). Moreover, ketoconazole has shown an efficacy of 95-100 % *in vitro* against *Aspergillus* species and *C. albicans* (Ho *et al.* 2006, Munguia & Daniel 2008).

On the other hand, the MIC of ketoconazole was much higher (1.25 mg/ml) on *R. mucilaginosa* (from cow), *A. niger* (from buffalo) and *R. mucilaginosa* and *S. brevicaulis* (from mares) while it was 5 mg/ml on *A. niger* (from cow) and *C. cladosporioides* (from mare). In this respect, the MIC of ketoconazole ranged between 0.5-2.0 µg/ml for isolates of clinical specimens origin of *R. rubra* (Barchiesi *et al*. 2000) and 1->16 µg/ml for *S. brevicaulis* (Aguilar *et al*. 1999) using a broth microdilution method.

Miconazole (Miconaz, one of the imidazole derivatives) was active on all fungi tested at 10 mg/ml concentration. Its MIC on *C. tropicum* and *E. nidulans* (from buffaloes), *C. albicans* and *P. chrysogenum* (from mares) was 1.25 mg/ml, however for *A. alternata, A. flavus* and *P. chrysogenum* (from cows) and *C. cladosporioides* and *S. brevicaulis* (from mares), the MIC was 2.5 mg/ml. On the other hand, the MIC of miconaz on *A. strictum* (from cow) and *A. fulvescenes, A. flavus, A. niger* and *C. albicans* (from buffaloes) increased to 5 mg/ml. In this respect, Sirohi and Khar (2000) stated that the antifungal drug miconazole had no much higher value, where

only 29.27% of the 41 isolates tested (related to *A. niger*, 10 of 17, and *C. albicans*, 2 of 2 were sensitive to 600 µg/ml concentration and its MIC on *C. albicans* and *A. niger* was 300 µg/ml and 150 µg/ml respectively. Aller Gancedo (1978) reported that some species of *Candida* were sensitive to miconazole as the MIC on all strains was 6.54 µg/ml using serial dilutions in a liquid medium. During their study of the *in vitro* susceptability of 75 clinical isolates of *C. albicans* to miconazole, Carrillo-Munoz *et al*. (2002) found that 9 strains were resistant, 15 were of intermediate susceptability and 51 were susceptible to miconazole. On the other hand, five isolates of *S. brevicaulis* from clinical specimens tested by Aguilar *et al*. (1999) displayed moderate susceptability to miconazole, with MIC ranging from 4->16 µg/ml using broth microdilution method. Also, MIC of imidazole derivatives (including miconazole) for *C. albicans, A. flavus, A. niger* and *E. nidulans* and *Penicillium* spp. were measured with liquid and solid media under a variety of experiment conditions and it was concluded that the concept that imidazole antifungals act similarly on all types of fungi should be applied with caution (Odds 1980).

Fluconazole (fungican) at 10 mg/ml affected all fungi tested, while it had no effect at 1.25 mg/ml. The MIC of fluconazole on *A. strictum* (from cow), *A. fulvescens, A. flavus, C. albicans, C. tropicum* and *E. nidulans* (from buffaloes) and *C. albicans* and *C. cladosporioides* (from mares) was 2.5 mg/ml. However, its MIC for *A. alternata, A. flavus* and *A. niger* (from cows), *A. niger* (from buffalo) and *P. chrysogenum* (from mare) was 5 mg/ml, while for *P. chrysogenum* and *R. mucilaginosa* (from cows) and *R. mucilaginosa* and *S. brevicaulis* (from mares) was 10 mg/ml (Table 2). In this respect, fluconazole was found to be active against *C. albicans* from clinical specimens with MICs ranging from 0.25 to 16 µg/ml by macrodilution method (Garg *et al*. 2006), from 0.12 to >128 µg/ml by broth microdilution tests (Pfaller *et al*. 2004) and from ≤0.125 to >64 µg/ml (Barchiesi *et al*. 2000) or 0.016 to 256 µg/ml by E test strips (Mallie *et al*. 2005). The in vitro study of Corrillo-Munoz *et al*. (2002) on susceptibility of 75 clinical isolates of *C. albicans* to fluconazole, revealed that 6 strains were resistant, 4 were intermediate susceptible and 65 were susceptible to fluconazole using the agar diffusion method. Fluconazole was also found to be active against *R. rubra* from clinical specimens and the MIC was ≥64 µg/ml using a broth microdilution method (Barchiesi *et al*. 2000), however in our study the MIC for *R. mucilaginosa* was 10 mg/ml where disc diffusion method was used.

Isolates of *S. brevicaulis* of clinical origin tested by Aguilar *et al*. (1999) (5 isolates) and Carillo-Munoz *et al*. (2005) (19 out of 20 isolates) were highly resistant to fluconazole with MIC being >64 µg/ml by microdilution method and 25 µg by tablets. However, our isolate of *S. brevicaulis* was more resistant and the MIC was much higher (10 mg/ml).

Betadine (povidone-iodine) antiseptic solution was effective on all fungal isolates tested *in vitro* at 100 mg/ml, while it had no effect on these fungi at lower concentrations (12.5 and 25 mg/ml). For *A. flavus, C. albicans* and *E. nidulans* (all from buffaloes) and *C. cladosporioides* and *P. chrysogenum* (from mares), the MIC was 50 mg/ml. For *A. strictum, A. alternata, A. flavus, A. niger, P. chrysogenum* and *R. muscilaginosa* (from cows), *A. fulvescenes, A. niger* and *C. tropicum* (from buffaloes) and *C. albicans, R. mucilaginosa* and *S. brevicaulis* (from mares), the MIC of betadine was 100 mg/ml. In this respect, Theraud *et al*. (2004) in their study on antiseptic betadine 10% (= 100 mg/ml) proved its effeciency as fungicidal for the five isolates they tested (3 clinical: *C. albicans, Cryptococcus neoformans* and *R. rubra* and 2 environmental: *C. albicans* and *Cryptococcus uniguttulatus*). Kremlev and Banakova (1979) found also that iodinol (iodine and potassium iodide in polyvinyl alcohol) at 125 µg/ml concentration inhibited the growth of *C. albicans, Proactinomyces ligniteresi, Aspergillus fumigatus, Absidia corymbifera* and *Mucor pusillus*. However, the efficacy of betadine intrauterine infusions was studied on 112 repeat breeder Holstein cows (aged 3-7 years) with endometritis revealed good results for the recovery and the conception rates (Koujan *et al*. 1996). Also, intrauterine irrigation of infected genitalia with 5% v/v of Lugol's solution (5 gm iodine and 8 gm potassium iodide in 100 ml distilled water) revealed negative mycotic isolations (Osman and Abou Gabal, 1975). Also, Povidine iodine was active against species of *Candida, Penicillium, Aspergillus, Epidermophyton* and *Microsporum* at different concentrations (Jayaraja Kumar *et al*. 2009). It was also concluded that diluted Lugol's iodine was a useful treatment of repeat breeder cows (Mutiga 1978).

Griseofulvin at 12.5 up to 200 mg/ml has no inhibitory effect on all fungi tested. In accordance with the current results, Jand *et al*. (1978) found that griseofulvin at 25-250 µg/ml has no inhibitory effect on *C. albicans, C. stellatoides, C. parapsilosis, C. guillermondii* and *Saccharomyces* sp. Griseofulvin had also no inhibitory action on yeasts (Aller Gancedo 1978).From the available literature, griseofulvin

is only inhibitory against dermatophytes (Sandhu and Randhawa 1964, Karaca and Koc 2004).

It could be concluded that nystatin followed by terbinafine and ketoconazole had the highest effect on most isolated fungi from repeat breeder animals.

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